PATENT COOPERATION TREATY

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NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

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International filing date (day/month/year) 07 June 2000 (07.06.00)	**Priority date** (day/month/year) 11 June 1999 (11.06.99)
Applicant GOLAY, Josee et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

21 December 2000 (21.12.00)

[ ] in a notice effecting later election filed with the International Bureau on:

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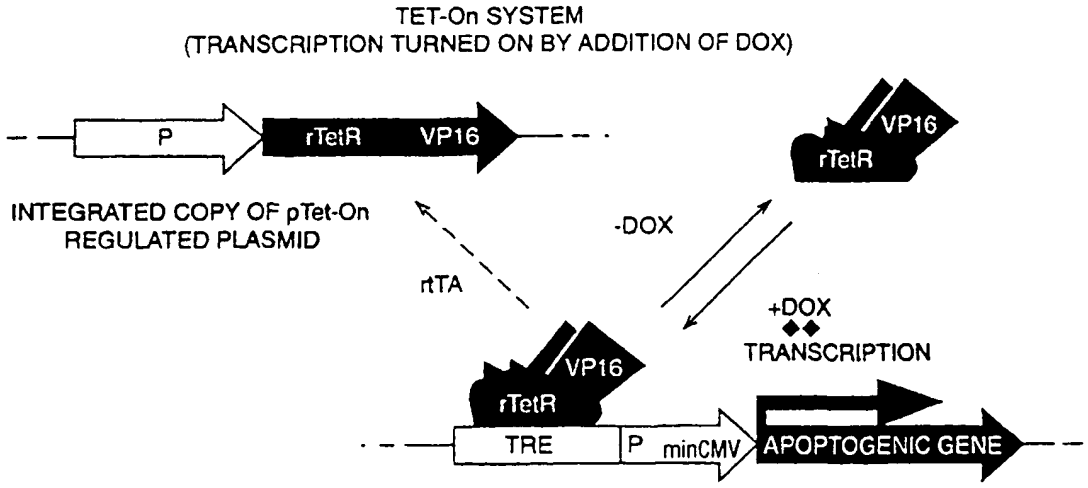
(54) Title: METHODS FOR SELECTING CELLS AND THEIR USES



(57) Abstract

Grafts, cells and tissues for use in transplantation, transgenic animals, methods of cell selection and various uses of such material. Use of induced apoptosis as a selectable negative marker for specific cell and tissue ablation avoiding local inflammatory response and "bystander effect" (e.g. in engrafted tissues and/or cells). Models for tissue-specific degenerative diseases and disorders and screening methods for compounds active against those diseases related to a cell/tissue specific depletion of static or expanding cell lines.

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INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/GB 98/00654

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/06 A01K67/027 G01N33/68

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K A01K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GB 2 294 945 A (STRINGER BRADLEY MICHAEL JOHN) 15 May 1996 see abstract see page 6, line 5 - line 10 see page 10, line 6 - page 23, line 20 ---	1-3,5-8
X	WO 97 07828 A (UNIV CALIFORNIA) 6 March 1997 see abstract see page 4, line 18 - page 7, line 19 see page 12, line 10 - page 19, line 30 ---	1-3,5
X,P	WO 97 45142 A (GENETIC THERAPY INC) 4 December 1997 see abstract see page 6 - page 17 * examples, claims * --- -/--	1-3,6

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search	Date of mailing of the international search report
7 September 1998	15/09/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Panzica, G

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/GB 98/00654

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 97 18307 A (SANDOZ AG ;BUEHLER THOMAS (CH); SANDOZ LTD (CH); SANDOZ AG (DE)) 22 May 1997 see abstract see page 1, line 1 - line 30	1
A	WO 96 14420 A (CANCER RES CAMPAIGN TECH ;AGRICULTURAL & FOOD RES (GB)) 17 May 1996 see abstract see page 3, line 15 - page 4, line 17	4,20

INTERNATIONAL SEARCH REPORT

ternational application No.
PCT/GB 98/00654

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 27 (partially)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

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2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claims Nos.: 27 (partially)

Claim 27 (an in vitro method of determining the effect of a deficit of a first class of cells on the characteristics of a second class) has neither reference to any of the precedent claims nor to the specific methods and embodiments of the application. These are namely the selectable positive marker and the induced apoptosis as negative marker and the targeted cells and tissues.
Therefore the teachings of claim 27 go broadly beyond the embodiments of the proposed invention.
Nonetheless, a search for claim 27 has been partially performed and has been limited according to the genes, cells and methods as defined previously in the application.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int tional Application No
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2294945 A	15-05-1996	AU 3812095 A	31-05-1996
		CZ 9701384 A	12-11-1997
		EP 0791051 A	27-08-1997
		WO 9614396 A	17-05-1996
		HU 77080 A	02-03-1998
WO 9707828 A	06-03-1997	AU 7018496 A	19-03-1997
		EP 0861092 A	02-09-1998
WO 9745142 A	04-12-1997	AU 3079697 A	05-01-1998
WO 9718307 A	22-05-1997	AU 7684896 A	05-06-1997
WO 9614420 A	17-05-1996	AU 3812395 A	31-05-1996

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Published:
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— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/76542 A1

(54) Title: USE OF ANTIBODIES AGAINST CD20 FOR THE TREATMENT OF THE GRAFT VERSUS HOST DISEASE

(57) Abstract: It is described the use of antibodies against exogenous surface antigens not present on normal human T lymphocytes for the preparation of compositions for the treatment of the graft versus host disease in patients who have received T lymphocytes transduced with such exogenous surface antigens.

USE OF ANTIBODIES AGAINST CD20 FOR THE TREATMENT OF THE GRAFT VERSUS HOST DISEASE

SUMMARY OF THE INVENTION

The present invention refers to the use of antibodies against exogenous surface antigens not present on normal human T lymphocytes for the preparation of compositions for the treatment of the graft versus host disease in patients who have received T lymphocytes transduced with such exogenous surface antigens.

The invention further relates to vectors for the transfection of human T lymphocytes with exogenous surface antigens and human T lymphocytes transduced with exogenous surface antigens..

BACKGROUND

The problem of the clinical relapses in patients with hematologic neoplasias (leukaemia and lymphomas) represents an increasingly important problem. A precise therapeutic role has been assigned for many years to the transplantation procedures with total bone marrow or with circulating purified precursors (J.O.Armitage, Bone marrow transplantation, New England Journal of Medicine, 1994, 330, 827-838). The clinical efficacy of such procedures is partially based upon a mechanism of immune recognition of the leukaemic cells of the host by the donor's T lymphocytes (GVL = Graft Versus Leukaemia) (M.Sykes, FASEB J., 10, 721-730, 1996). Nonetheless the transplants are characterised by many toxic effects including the immunologic reactivity of the donor's lymphocytes themselves against the normal tissues of the host (GVDH= Graft Versus Host Disease). In other words, the administration of T lymphocytes to the host shows clear benefits associated with severe risks and it is impossible to pharmacologically separate these two aspects.

Although standardised immunoselection techniques allow today the easy production of large quantities of purified donor's T lymphocytes for administration in order to induce in vivo the GVL effect, appropriate techniques to pharmacologically

induce the selective death of the administered T lymphocytes in a patient in order to eliminate the GVHD effect in the moment in which this is clinically needed are not yet available.

In the last years many polyclonal and monoclonal antibodies have been produced against human surface molecules; in many cases antibodies have been produced with the direct aim of killing in vivo a cell positive for that molecule so to be utilised in immunotherapy protocols; as an example, limiting to the B lymphoma area, efficacious antibodies have been produced and characterised against the CD20, CD19, CD40, CD22, CD52, CD38 molecules and yet others (P.S.Multani et al., J.Clin.Oncol., 16, 3691-3710, 1998). In some cases the antibodies directed against such molecules have shown in vivo cytotoxicity probably as they are able to activate the complement system on the surface of the target cell, as is the case with CD20, CD38, and CD52. In other cases antibodies have been conjugated with radioactive molecules to induce the target radiolysis, as is the case with CD20, Lym-1 and others. Other antibodies have been conjugated to toxins of bacterial or vegetable origin with the same aim, as is the case with CD19, CD40 and CD22. Other antibodies have been chimerised to allow a bispecificity so to bring two cells in close proximity, for example. Finally, for many of these antibodies engineered and/or humanised versions exist which allow to administer them in vivo reducing the risk of antigenicity and increasing their efficacy.

DISCLOSURE OF THE INVENTION

It has been now found that it is possible to effectively control the graft versus host disease problem by use of a method comprising the introduction of an exogenous surface antigen in the donor's T lymphocytes and the subsequent administration to the receiving patient of the antibodies directed against such exogenous antigen.

By exogenous antigen any surface antigen not present on normal T lymphocytes is meant, as is the case with the antigens expressed on the surface of B lymphocytes such as CD20, CD19, CD40, CD22, CD52 etc. etc. Obviously, the surface antigen will

be selected so as not not cause, following the reaction with the corresponding antibody, negative or unwanted effects at the level of the cellular populations which express constitutively the antigen.

It is particularly preferred the CD20 surface antigen of the human B lymphocytes against for which a humanised monoclonal antibody is commercially available (Rituximab ®, Roche) which is used in the treatment of B non Hodgkin lymphomas.

According to the invention, donor T lymphocytes are transduced by suitable techniques with the selected antigen and are then enriched through immunoaffinity methods before being injected to the receiving subject. In case the graft versus host disease develops, the antibody against the antigen is administered in order to inactivate in vivo the T lymphocytes by use, for example, of complement mediated cytotoxic mechanisms.

The antibody will preferably be monoclonal, more preferably it will be a humanised monoclonal antibody. Dosages and administration route will depend on many factors including overall health status, weight, sex and age of the patient. Generally the antibody will be administered by iv route in a dosage range from approximately 50 to approximately 500 mg/m^2 of body surface, one to three times a day until the almost complete disappearance of the circulating T lymphocytes.

The isolation of T lymphocytes has been described by Rambaldi et al., Blood, 91, 2189-2196, 1998.

The methods to transduce the T lymphocytes with the desired antigen are well known: as a reference see the review by Verma I.M. and Somia N. in Nature, 389, 239-242, 1997. In particular, suitable vectors can be used, such as retroviruses, adenoviruses, adeno associated viruses, herpesviruses, lentiviruses etc. etc.

Each of these vectors includes, in its turn, many different types of organisms: considering retroviruses, examples are amphotropic, ecotropic and xenotropic vectors. Furthermore many different packaging cell lines have been utilised in the years to optimise the production of such recombinant retroviruses and to guarantee better

handling and safety for the producers (I.M.Verma et al., Nature, 389, 239-242, 1997; M.A.Kay et al., Gene therapy, Proc. Natl. Acad. Sci.USA, 94, 12744-12746,1997).

Recently, also naked DNA has been introduced into target cells through conjugation with polycationic or liposomal complexes, electroporation, precipitation in salt buffers and other techniques.

Many different cell types have been targeted with genetic transfer: T and B lymphocytes, immature haematopoletic precursors, muscle cells, fibroblasts, hepatocytes and other cell types (I.M. Verma et al., Nature, 389, 239-242, 1997;M.A. Kay et al., Gene therapy, Proc. Natl. Acad. Sci. USA, 94, 12744-12746, 1997)

In the case of CD20 antigen, an amphotropic retrovirus has been used which derives from the Moloney murine leukaemia virus and is packaged in embryonic kidney human cells (293 T) engineerized to contain the retroviral structural elements on separate plasmids (Human Gene Therapy, 7, 1405-1413,1996). Such vectors, as well as the T lymphocytes transduced with the exogenous antigen, in particular the CD20+ T lymphocytes, are an object of the present invention.

After the genetic transfer the cells which express significantly the exogenous gene constitute only a minority of the total population. Selection procedures of the transduced cells are carried out, by use of exogenous genes which are able to give a selective advantage to the cell. The transduced cells can also be selected according to alternative methods such as FACS sorting with antibodies against the exogenous antigens (K.Phillips, et al., Nature Medicine, 2, 10, 1154-1155, 1996). Other methods are immunoaffinity columns or preadsorbed culture plates for the panning procedure, and the like.

Description of the figures

Figure 1: scheme of the plasmid LTR CD20 LTR;

LTR = long terminal repeat; pUC = plasmid origin of replication; Puro = gene which confers puromycin resistance; PGK1 = promoter of the phosphoglyceraldehyde kinase; EBNA1 and OriP = elements derived from the EBV virus for the episomal

replication; AmpR = gene for the ampicillin resistance.

Figure 2: infection of the CEM cell line with CD20 and immunoselection.

Left panel A: CEM cell line after virus infection, analysed at the cytofluorimeter with a fluorescent control IgG1 antibody.

Central panel B: the same population analysed with a fluorescent anti CD20 antibody.

Right panel C: the same population after immunoselection on affinity columns, analysed with a fluorescent anti CD20 antibody.

Figure 3: infection of human fresh T lymphocytes with CD20 virus

Left panel A: after the infection the lymphocytes are labelled with PE IgG2a and FITC IgG1 control antibodies.

Right panel B: same population is labelled with anti CD20 PE and anti CD3 FITC antibodies. In the shown case 23% of the cells are double positive.

The following examples illustrate the invention in greater detail

Example 1

Construction of the plasmid LTR CD20 LTR

A 913 nt fragment from the human CD20 cDNA containing the entire coding sequence has been obtained by PCR from the plasmid pCMV CD20 (Becker et al., Science, 249, 912-915, 1990).

For the amplification, 40 ng of plasmid were brought in a final reaction volume of 100 µl in 10 mM KCl, 10 mM (NH4)2SO4, 20 mM Tris HCl, pH 8.75, 2 mM $MgSO_4$, 0.1% Triton X-100, 100 µg/ml BSA, in the presence of 0.8 µl of a solution of 2.5 mM dNTP, 500 ng of primer "sense" (CGGGATCCAAAATGACAACACCCAGAAATTC), 500 ng of primer "antisense" (CGGGATCCTTAAGGAGAGCTGTCATTTTCT) and 5U Pfu DNA Polymerase from Stratagene (La Jolla, CA, USA). The reaction was carried out for 26 cycles in the cycler following this scheme: 1' at 95° C, 1' at 60°C and 2' at 72°C. At the end of the reaction 100 µl of a 25:24:1 phenol chloroform and isoamyl alcohol solution were

added and after extraction, DNA was precipitated overnight at 20°C in the presence of ethanol. After centrifugation, DNA was resuspended in 100 µl water and then subcloned in the pMOS vector (Amersham Italia, srl, Italy) according to the manufacturer's instructions contained in the kit "pMOS blunt ended cloning kit". The resulting recombinant plasmid was amplified and sequenced, then digested with BamHI whose recognition site (G/GATCC) was present in both PCR primers' ends. Therefore the fragment was subcloned in the BamHI site of the retroviral vector PINCO VUOTO. The retroviral vector PINCO VUOTO had been previously obtained following excision with EcoRI and NotI of a 1441 bp fragment containing the CMV promoter (Cytomegalovirus) and the EGFP (enhanced green fluorescent protein) gene from the plasmid PINCO (F.Grignani e al., Cancer Res., 58, 14-19, 1998). After excision of the EcoRI-NotI fragment, the plasmid was closed after end blunting with Klenow fragment and called PINCO VUOTO. Such retroviral vector is now of 11448 bp in length.

The recombinant between PINCO VUOTO and the CD20 cDNA was called LTR-CD20-LTR and sequenced to check the cloning and the integrity of the CD20 cDNA as well as the absence of stop codons upstream the first ATG (Fig.1).

The construct LTR-CD20-LTR is therefore made of, for the retroviral portion, the LTR derived from the Moloney murine leukaemia virus (MoMLV), other retroviral sequences derived from the Moloney virus, the CD20 cDNA in the BamHI site and the second LTR as detailed in annexed Fig.1. The rest of the plasmid is identical to the PINCO plasmid (F.Grignani et al., Cancer Res., 58, 14-19, 1998) which contains, as shown in the figure, EBNA-1 and OriP elements from the Epstein Barr virus, the origin of replication (pUC) and the gene for the ampicillin resistance, as well as a gene for the puromycin resistance under the control of PGK-1 promoter.

Example 2

Transfection of the LTR-CD20-LTR plasmid in the packaging cells

In order to produce retroviruses, the packaging cell Phoenix-Ampho was

transfected with the LTR-CD20-LTR plasmid.

The Phoenix-Ampho cells are derived from the human embryonic kidney 293 cell line following several modifications; initially they were transfected with the E1A gene from adenovirus and then transfected with two separate plasmids coding for the structural genes gag and pol from Moloney MLV under the control of Rous sarcoma virus promoter and the env gene from Moloney MLV under the control of cytomegalovirus promoter.

1.5 x 10^6 cells were plated on day -1 in a Petri dish of 10 cm diameter in 10 ml DMEM medium (Gibco, Seromed, Berlin, Germany) added with 10% FCS (Hiclone Laboratories, Steril System, Logan, UK) and kept in 5% CO_2 incubator at 37°C. On day 0 16 µl chloroquine were added (stock solution 25 mM in PBS) and after 10' 1 ml solution of 10 µg plasmid DNA was added. To obtain such DNA solution, 500 µl of a solution 2X HBS (50 mM HEPES, pH 7.05, 10 mM KCl, 12 mM Dextrose, 280 mM NaCl, 1.5 mM Na_2HPO_4 (FW 141.96)) were added in a 15 ml conic tube. Subsequently, in a second 15 ml tube, 500 µl of a solution with 10 µg DNA, 61 µl CaCl 2M and sterile water were prepared. After that, the DNA mixture was added dropwise in the first tube and the obtained precipitated was then added to the cells.

After 8 hours the medium was replaced with 10 ml of fresh DMEM.

On day +1 the medium was replaced with 5 ml fresh RPMI 1640 medium added with 10% FCS.

On day +2 the infection was carried out by removing the 5 ml of medium containing the retroviruses released during the culture.

Example 3

Infection of the CEM cell line with the LTR-CD20-LTR retrovirus

1 x 10^6 human T lymphoblastoid CEM cells growing in suspension in RPMI 1640 medium supplemented with 10% FCS and glutamine, were pelleted by spinning at 1200 rpm for 8' in a flat bottom well of a 24 wells plate (Falcon, Becton Dickinson and Company, NY). After removal of the supernatant, 1 ml of the viral supernatant

was added by filtration through 0.45 μm filters (Millipore Corporation Bedford, MA) in the presence of 1 μl Polybrene (stock solution 4 mg/ml in PBS).

The plate was then centrifuged for 45' at 1800 rpm at room temperature and then the supernatant was removed and replaced with 1 ml fresh RPMI 1640 added with 10% FCS and subsequently incubated for additional 6 hours.

At the end of the incubation the infection procedure was repeated a second time using a different Petri dish of packaging cells previously prepared.

Example 4

FACS analysis of CD20+ CEM

CEM cells following retroviral infection with LTR-CD20-LTR were kept in the incubator and normally grown in RPMI 1640 medium added with 10% FCS. After 2 days the CEM cells could already be assayed by immunofluorescence analysis for the presence of the CD20 marker on the surface.

0.1 x 10^6 cells were transferred in an 1.5 ml Eppendorf tube, spun at 4,000 rpm for 3', resuspended in 50 μl of a solution of fluorescent anti CD20 1F5 antibody (Becton Dickinson) and kept for 30' at 4°C. At the end, 500 μl of a solution 0.9% NaCl, 5% FCS, 0.02% Na Azide were added and cells were spun at 4,000 rpm for 5'. After that, the sample was resuspended in 100 μl of PBS solution containing 1% formaldehyde and then kept at 4°C until reading at the fluorocytometer.

In many experiments this infection procedure always gave CEM CD20+ cells in varying percentages from 30 to 60%, while the non infected cell line was completely negative for the CD20 expression (as an example see Fig. 2, central panel, showing a CEM population which became by 40% CD20+.).

Example 5

Immunoaffinity separation

CEM cells infected with LTR-CD20-LTR virus after two days of culture cuold be enriched in the CD20+ population by immunoaffinity columns. To this purpose cells were first incubated for 30' at 4°C with the anti CD20 antibody clone 1F54, then

washed three times with PBS and 2.5% human serum albumin, finally incubated for additional 30' at 4°C with a solution of microbeads coated with a goat anti mouse IgG antibody (Milteny Biotech, Bergish-Gladbach, Germany).

Finally the cells were resuspended in medium RPMI 1640 and selected through passage on XS+ column in the SuperMACS system (Milteny Biotech). Then the column was eluted with physiologic solution added with 2.5% albumin and the column was removed from the SuperMACS and washed in order to recover the positive fraction.

The positive fraction was further analysed at the cytofluorimeter following cell labelling according to the direct immunofluorescence procedure previously described.

The percentage of CD20+ cells at the end of this procedure has always been above 90%. As an example see Fig.2, right panel, in which a CEM population is shown after enrichment by immunoaffinity which is CD20+ positive at 98%.

At the end the CEM CD20+ population was grown in suspension and expanded in medium RPMI 1640 added with 10% FCS in incubator. At regular intervals this population was studied for the expression of the CD20 marker on the surface thus showing the stability of the marker for more than two months and the positivity on more than 90% of the selected cells.

Example 6

Infection with the LTR-CD20-LTR virus of peripheral fresh T lymphocytes

Heparinised total blood was stratified over Ficoll and centrifuged for 30' at 1,500 rpm at room temperature. The cells collected at the interface were washed with PBS and spun at 1,500 rpm for 10' at room temperature, then two further times at 1,000 rpm for 10' at room temperature and finally resuspended in RPMI 1640 with 10% FCS at 1 x 10^6 / ml in 24 wells plates with flat bottoms, aliquoting 2 ml of cell suspension per well in the presence of PHA (Murex) at 1 µg/ml at 37°C and 5% CO_2 for one night.

The second day human recombinant IL-2 was added (Proleukin, Chiron Italia,

Milan, Italy) at the final concentration of 100U/ml.

At the third day, after washing and cell countings, 1 x 10^6 cells were infected in 1 ml of medium in one flat bottom well in a 24 wells plate. After spinning at 1,200 rpm for 10', the supernatant was removed and replaced with 1 ml filtered virus in the presence of polybrene and subsequent spinning for 45' at 1,800 rpm at room temperature as from the above referred protocol.

At the end, the viral supernatant was removed and replaced with complete medium for 6 hours incubation and then the spin infection procedure was repeated. After that, cells were resuspended in complete medium in the presence of Il-2 and left to stand in the incubator overnight.

The entire procedure was repeated for the following two days and finally the cells were kept in culture for two additional days in incubator.

Then the cells were labelled with monoclonal antibodies anti CD20 FITC, anti CD3 PE, anti CD4 PE, and anti CD8 FITC (Becton Dickinson) with the same procedure described above and then analysed at the cytofluorimeter.

Many experiments on normal donors show that a varying percentage from 5% to 25% of CD3+ T lymphocytes acquires the CD20 marker in double fluorescence analysis. One such experiment is shown in Fig. 3, in this specific case 23% CD3/CD20 double positivity having been attained.

Example 7

Study of the lysis induced by antibody and complement in populations of fresh human T lymphocytes after CD20 gene transduction.

2 x 105 transduced lymphocytes were aliquoted in 10 ml round bottomed tubes in 500 µl of RPMI 1640 medium added with 10% heat inactivated foetal calf serum. Then the Rituximab antibody was added to the final concentration of 350 g/ml and rabbit Pel freeze complement at final 10%.

Alternatively, human AB serum at the final 30% concentration can be added as a source of complement. Cells were left for one hour at 37°C in a thermostatized water

bath with continuous shaking. The cell suspension was added with an equal volume of 1X solution of acridine orange in PBS (stock 100 X solution consisting of 30 mg in 100 ml distilled water) and the cell suspension was evaluated at the cytofluorimeter: the living cells emit green fluorescence and were counted as percentage on the total population analysed. With this quick method, the killing efficiency of Rituximab® on the CD20+ cells could be assessed, comparing the percentages of double positive CD3/CD20 cells in the different studied populations and the percentages of dead cells after Rituximab® addition. As shown in the Table, the control populations were the same cells exposed to the antibody alone or to complement alone. Data shown in the table prove that one hour exposure to Rituximab® induces almost 90% death of the CD3/CD20 + cells.

Table: Complement-dependent cytotoxicity of CD20 transduced fresh human T lymphocytes

		% specific lysis		
	% CD3/CD20+ lymphocytes	Rituximab® Alone	Complement alone	Rituximab® Plus Complement
Donor 1	30	0	14	33
Donor 2	23	0	11	35
Donor 3	15	0	5	18

The lysis percentage was determined at the FACS following staining with acridine.

CLAIMS

1. The use of antibodies against exogenous surface antigens not present on human normal T lymphocytes, for the preparation of compositions for the treatment of the graft versus host disease in patients who have received T lymphocytes transduced with such exogenous surface antigens.

2. The use according to claim 1, wherein antibodies against the CD20 surface antigen and lymphocytes transduced with the CD20 antigen are used.

3. The use according to claim 2, wherein the anti CD20 antibody is a humanised monoclonal antibody.

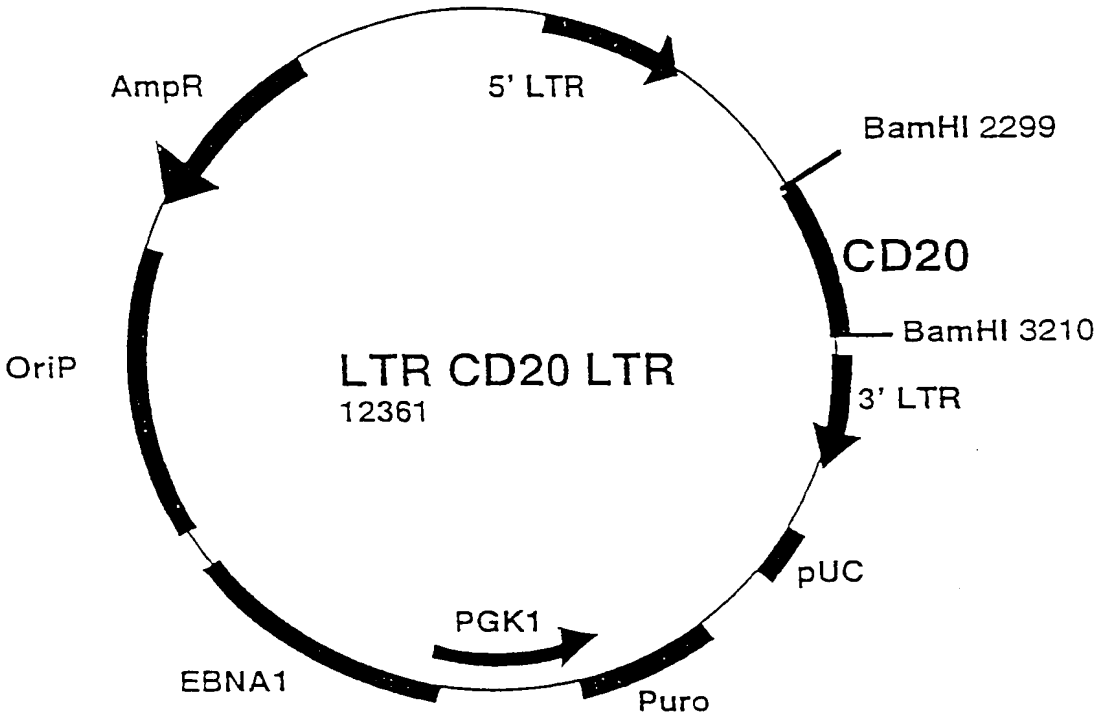
4. Vectors for the transfection of human T lymphocytes with exogenous surface antigens.

5. Vectors according to claim 4 including the gene coding for the human CD20 antigen.

6. Human T lymphocytes transduced with exogenous surface antigens.

7. T lymphocytes according to claim 6 transduced with human CD20 antigen.

FIG 1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Puro
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LTR CD20 LTR
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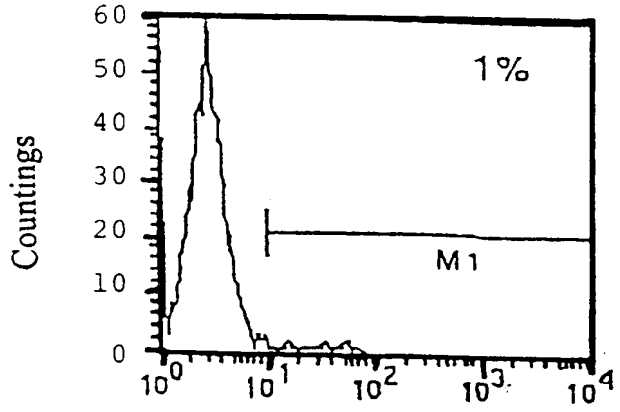
FIG 2



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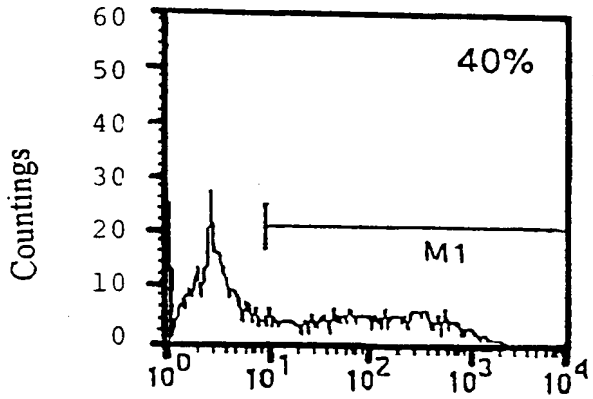

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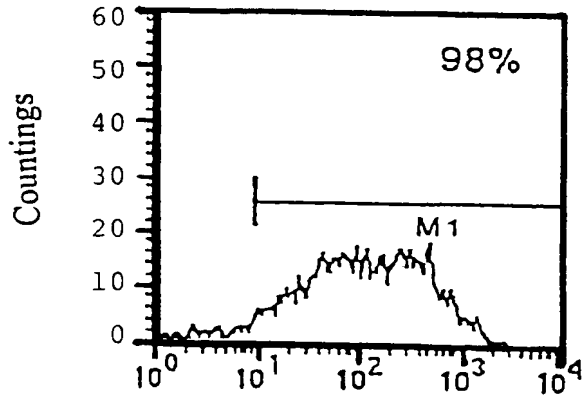

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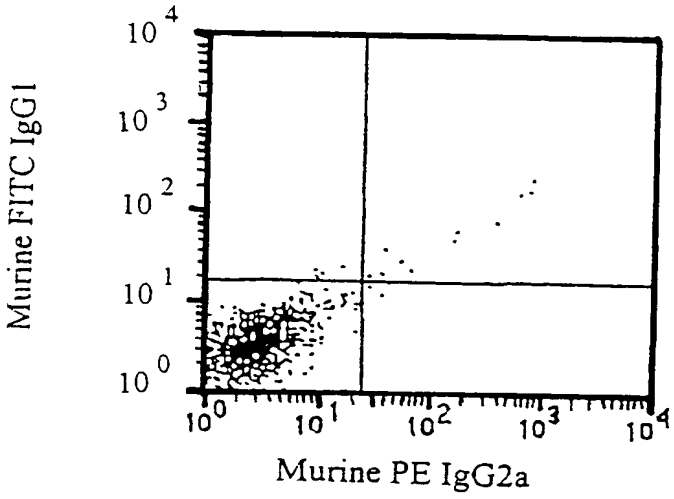
CD20 FITC

FIG 3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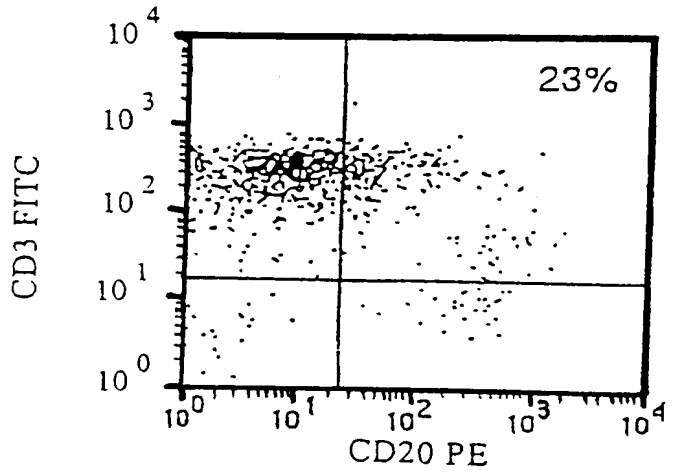
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B

INTERNATIONAL SEARCH REPORT

Int[redacted]pplication No
PCT/EP 00/05212

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/395 A61K35/28 C12N5/10 C12N15/85 A61P43/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
MEDLINE, WPI Data, EPO-Internal, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VERZELETTI SIMONA ET AL: "Herpes simplex virus thymidine kinase gene transfer for controlled graft-versus-host disease and graft-versus-leukemia: Clinical follow-up and improved new vectors." HUMAN GENE THERAPY, vol. 9, no. 15, 10 October 1998 (1998-10-10), pages 2243-2251, XP000946824 ISSN: 1043-0342 abstract page 2244, left-hand column, line 18-37,51-56 page 2244, right-hand column, line 25-54 ---	4,6
A	WO 97 45142 A (GENETIC THERAPY INC) 4 December 1997 (1997-12-04) page 6, paragraph 2 -page 7, line 3 --- -/--	1-7

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 October 2000	20/10/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Covone, M

3

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 42824 A (CELLFACTORS PLC ;DAVIES ALISON MIRIAM (GB)) 1 October 1998 (1998-10-01) page 2, line 8-15 page 8, line 14-19 claims 1-13	1-7
A	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; August 1998 (1998-08) MCLAUGHLIN PETER ET AL: "Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: Half of patients respond to a four-dose treatment program." Database accession no. PREV199800407092 XP002149247 abstract & JOURNAL OF CLINICAL ONCOLOGY, vol. 16, no. 8, August 1998 (1998-08), pages 2825-2833, ISSN: 0732-183X	1-7
P,X	INTRONA MARTINO ET AL: "Genetic modification of human T cells with CD20: A strategy to purify and lyse transduced cells with anti-CD20 antibodies." HUMAN GENE THERAPY, vol. 11, no. 4, 1 March 2000 (2000-03-01), pages 611-620, XP000946823 ISSN: 1043-0342 the whole document	1-7

INTERNATIONAL SEARCH REPORT

Information on patent family members

In ... Application No
PCT/EP 00/05212

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9745142 A	04-12-1997	AU 719930 B	18-05-2000
		AU 3079697 A	05-01-1998
		CA 2255941 A	04-12-1997
		EP 0977595 A	09-02-2000
		NO 985522 A	25-01-1999
WO 9842824 A	01-10-1998	AU 6736598 A	20-10-1998

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(19) World Intellectual Property Organization
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(43) International Publication Date
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(10) International Publication Number
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(21) International Application Number: PCT/EP00/05212

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(71) Applicant *(for all designated States except US)*: **CONSIGLIO NAZIONALE DELLE RICERCHE** [IT/IT]; P.le Aldo Moro 7, I-00185 Roma (IT).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **GOLAY, Josee** [CH/IT]; Laboratorio di Ummunoematologia Molecolare, Istituto Ricerche Farmacologiche Mario Negri, Via Eritrea 62, I-20157 Milano (IT). **INTRONA, Martino** [IT/IT]; Laboratorio di Immunoematologia Molecolare, Istituto Ricerche Farmacologiche Mario Negri, Via Eritrea 62, I-20157 Milano (IT). **RAMBALDI, Alessandro** [IT/IT]; Divisione di Ematologia, Ospedali Riuniti, Largo Barozzi 1, I-24128 Bergamo (IT). **BIONDI, Andrea** [IT/IT]; Centro Ricerche Tettamanti, Clinica Pediatrica Università di Milano-Bicocca, Via Donizetti 106, I-20052 Monza (IT).

(74) Agents: **MINOJA, Fabrizio** et al.; Bianchetti Bracco Minoja SRL, Via Rossini 8, I-20122 Milano (IT).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/76542 A1

(54) Title: USE OF ANTIBODIES AGAINST CD20 FOR THE TREATMENT OF THE GRAFT VERSUS HOST DISEASE

(57) Abstract: It is described the use of antibodies against exogenous surface antigens not present on normal human T lymphocytes for the preparation of compositions for the treatment of the graft versus host disease in patients who have received T lymphocytes transduced with such exogenous surface antigens.

FIG 1

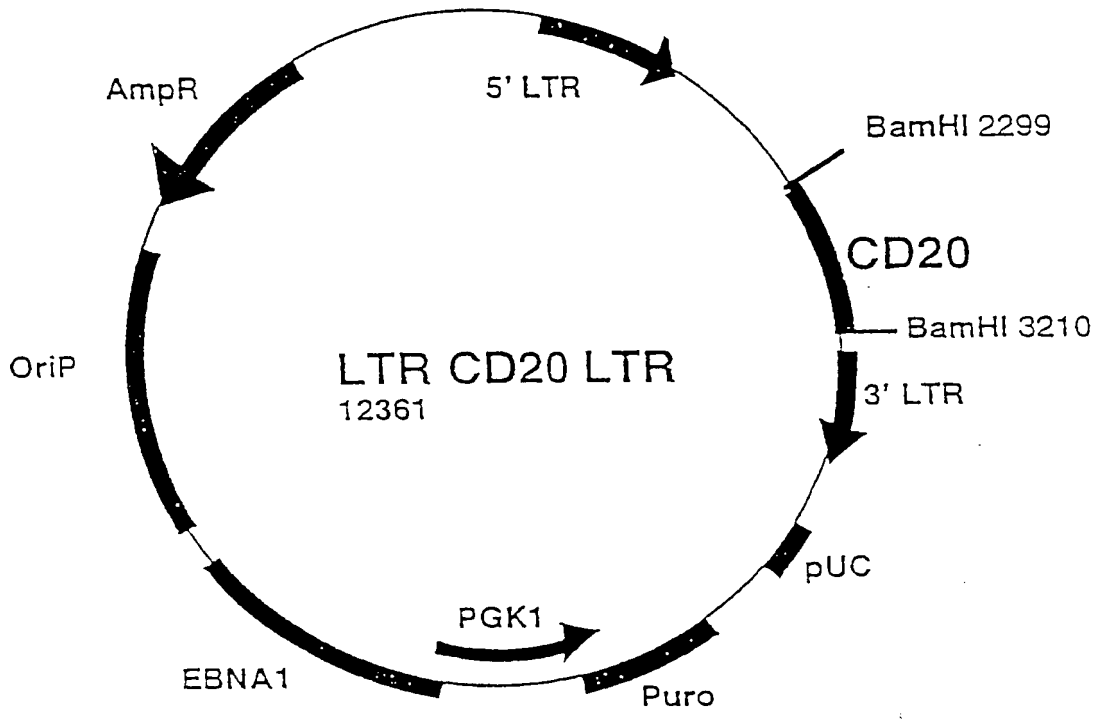
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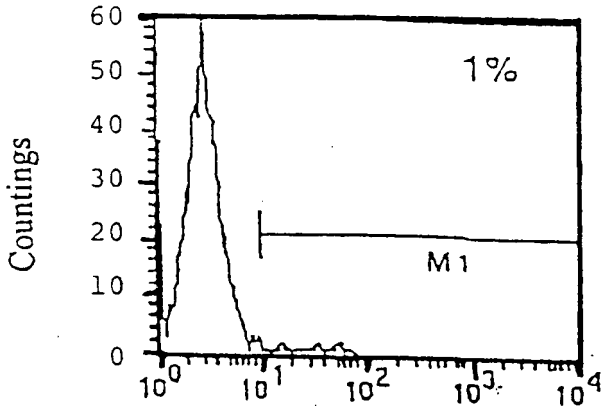
FIG 2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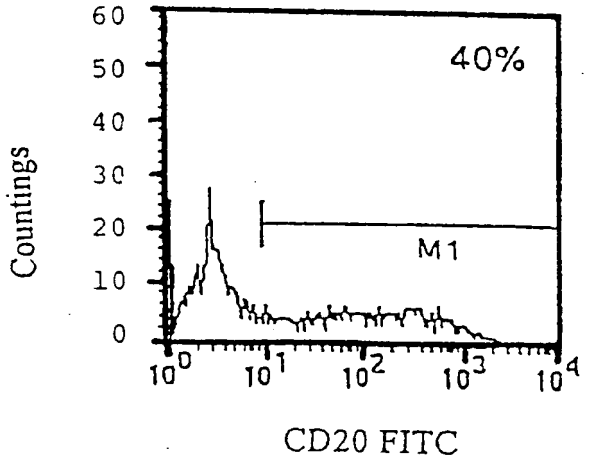
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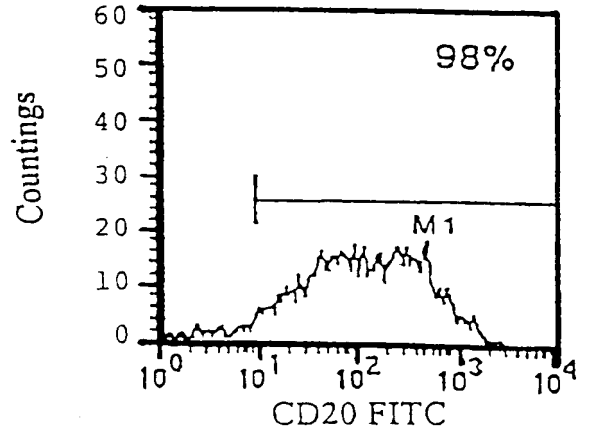
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C

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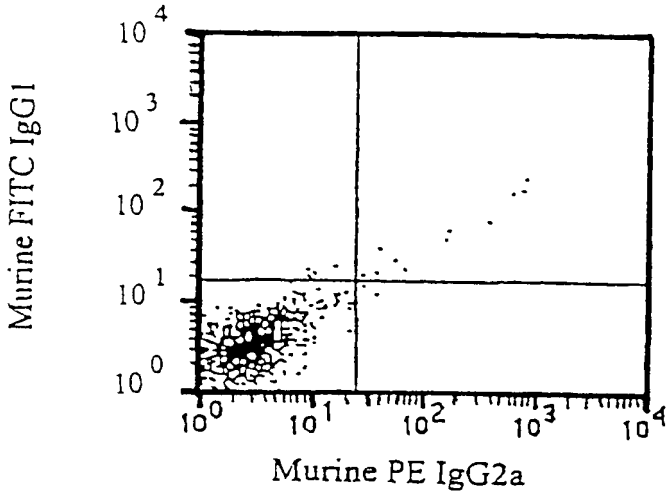
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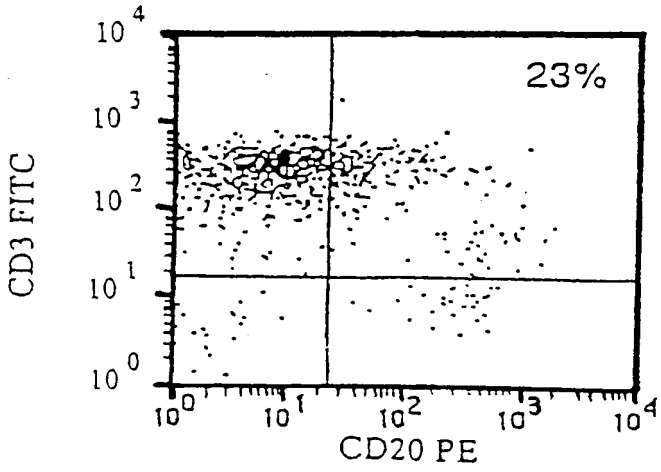
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INTERNATIONAL SEARCH REPORT

Int[illegible]ional Application No
PCT/EP 00/05212

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/395 A61K35/28 C12N5/10 C12N15/85 A61P43/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
MEDLINE, WPI Data, EPO-Internal, PAJ, BIOSIS

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A	WO 97 45142 A (GENETIC THERAPY INC) 4 December 1997 (1997-12-04) page 6, paragraph 2 -page 7, line 3 --- -/--	1-7

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 October 2000	20/10/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Covone, M

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 42824 A (CELLFACTORS PLC ;DAVIES ALISON MIRIAM (GB)) 1 October 1998 (1998-10-01) page 2, line 8-15 page 8, line 14-19 claims 1-13	1-7
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P,X	INTRONA MARTINO ET AL: "Genetic modification of human T cells with CD20: A strategy to purify and lyse transduced cells with anti-CD20 antibodies." HUMAN GENE THERAPY, vol. 11, no. 4, 1 March 2000 (2000-03-01), pages 611-620, XP000946823 ISSN: 1043-0342 the whole document	1-7

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 00/05212

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9745142 A	04-12-1997	AU 719930 B	18-05-2000
		AU 3079697 A	05-01-1998
		CA 2255941 A	04-12-1997
		EP 0977595 A	09-02-2000
		NO 985522 A	25-01-1999
WO 9842824 A	01-10-1998	AU 6736598 A	20-10-1998

PATENT COOPERATION TREATY

PCT

14

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
SCB566PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/05212	07/06/2000	11/06/1999
International Patent Classification (IPC) or national classification and IPC A61K39/395		
Applicant CONSIGLIO NAZIONALE DELLE RICERCHE et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 1 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
21/12/2000	13.08.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer BROCHADO GARGANTA, M Telephone No. +49 89 2399 8935

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/05212

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-11 as originally filed

Claims, No.:

1-7 as received on 24/07/2001 with letter of 20/07/2001

Drawings, sheets:

1/3-3/3 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-3, 5-7
	No:	Claims	4
Inventive step (IS)	Yes:	Claims	1-3,6-7
	No:	Claims	4,5
Industrial applicability (IA)	Yes:	Claims	1-7
	No:	Claims	

2. Citations and explanations
see separate sheet

Re Item I
Basis of the report

1. The amendments filed on 23.07.2001 do not introduce additional subject-matter, which extends beyond the content of the application as filed. Therefore, the amendments meet the requirements of Article 34(2)(b) PCT.

Re Item V
Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following documents:

   (A) Verzeletti Simona et al.: Human Gene Therapy, vol. 9, no. 15, 10 October 1998, pages 2243-2251

   (B) Database Biosis [Online] BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; August 1998 (1998-08) Mclaughlin Peter et al.:Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: Half of patients respond to a four-dose treatment program.' & JOURNAL OF CLINICAL ONCOLOGY, vol. 16, no. 8, August 1998 (1998-08), pages 2825-2833

   (C) Introna Martino et al.: 'Genetic modification of human T cells with CD20: A strategy to purify and lyse transduced cells with anti-CD20 antibodies.' Human Gene Therapy, vol. 11, no. 4, 1 March 2000 (2000-03-01), pages 611-620

2. Document C, cited in the Search Report as intermediate document, is not to be considered as state of the art, as the date of priority is validly claimed for the relevant parts of the present application.

3. Novelty

3.1 The subject-matter of claims 1-3, 6-7, relating to the use of antibodies for the treatment of the graft versus host disease and human T lymphocytes, is new in the sense of Article 33(2) PCT, because this subject-matter being related to antigens expressed on the surface of B-lymphocytes and not present on human normal T lymphocytes, is not disclosed in the prior art.

3.2 Claim 4 relates to a vector for the transfection of human T-lymphocytes with antigens expressed on the surface of B-lymphocytes and not present on human normal T-lymphocytes. this subject-matter is already disclosed in document A and therefore, claim 4 is not new in the sense of Article 33(2) PCT.

Document A discloses several vectors for the transfection of human T-lymphocytes having a cell surface selectable marker. Such improved vectors would overcome the difficulties and problems that arise after administration of a therapeutic infusion of donor lymphocytes to patients suffering from severe graft-versus-host disease (see abstract). The fact that ,the vectors of the present application are "suitable for" tranfection of human T-lymphocytes with antigens expressed on the surface of B-lymphocytes and not present on human normal T-lymphocytes, does not change the vector as such. Moreover, this vector is not characterised by means of technical features.

The additional features of claim 5 are not disclosed in document A and therefore claim 5 is novel (Article 33(2) PCT).

4. Inventive step

4.1 Document B discloses the **use of antibodies against CD20 antigen** for the preparation of compositions for the **treatment of lymphoma** (see abstract).

The differences between the subject-matter of claim 1 and the disclosure of document B, is the fact that the antibodies are used for treating **graft versus host disease** in patients who have received T lymphocytes transduced with an exogenous surface antigen and that these **antigens are not present on human normal T lymphocytes**.

Leukaemia and **lymphomas** belong to the group of haematologic neoplasias. A therapeutic role for these disorders has been assigned for **many years** to the transplantation procedures with total bone marrow or with circulating purified precursors, as **T lymphocytes**. The clinical efficacy of such procedures is based upon a mechanism of immune recognition of the leukaemia cells of the host by the donor's T Lymphocytes (Graft Versus Leukaemia). Transplants are characterised by many toxic effects including the immunologic reactivity of the donor's lymphocytes themselves against the normal tissues of the host (**Graft versus host disease**).

Document A refers that **graft versus host disease,** associated with the therapeutic infusion of donor lymphocytes, transfected with retroviral vectors carrying the HSV-tk suicide gene and a cell surface marker, after allogenic marrow transplantation, can be efficiently controlled by expression of the herpes simplex virus thymidine kinase (see abstract).

There is no suggestion in document A to use surface antigens and antibodies against them to control the activity of donor T-lymphocytes. Even combining the teachings of A and B, the skilled person would not have arrived at the claimed invention in an obvious manner. There is no reference in document B to either transduction of T-lymphocytes with exogenous CD20 or to a method for controlling GvHD using CD20-transduced T-lymphocytes. Moreover, in document B the cells involved are B-lymphocytes, not T-lymphocytes, and a person skilled in the art would have refrained from using an antibody specific for a certain antigen to treat a disease in which cells that do not express the same antigen are involved.

Thus, even combining the disclosures set out in documents A and B, the skilled person would not arrive in an obvious way to the features of claim 1. Therefore, claim 1 is based on an inventive concept as required by Article 33(3) PCT.

4.2 Claim 6 relates to human T lymphocytes transduced with antigens expressed on the surface of B-lymphocytes and not present on human normal T-lymphocytes. Considering the reasoning given in 4.1, this claim is also considered to be based on an inventive concept (Article 33(3) PCT).

For the same reasons, dependent claims 2, 3 and 7, relating to anti CD20 antibody,

are also inventive as the claims on which they depend are considered to be based on an inventive concept (Article 33(3) PCT).

4.3 The additional feature of claim 5, also relating to anti CD20 antibody, is known from document B (see abstract) and therefore this claim is not based on an inventive concept (Article 33(3) PCT). In fact, it would be obvious for the skilled person to combine the disclosures of documents A and B and arrive in this way to the features of claim 5, as both documents relate to the treatment of lymphoma or of graft versus host disease.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
SCB566PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/05212	07/06/2000	11/06/1999
International Patent Classification (IPC) or national classification and IPC A61K39/395		
Applicant CONSIGLIO NAZIONALE DELLE RICERCHE et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 1 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
21/12/2000	13.08.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer BROCHADO GARGANTA, M Telephone No. +49 89 2399 8935

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/05212

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-11	as originally filed				

Claims, No.:

1-7	as received on	24/07/2001	with letter of	20/07/2001	

Drawings, sheets:

1/3-3/3	as originally filed				

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-3, 5-7
	No:	Claims	4
Inventive step (IS)	Yes:	Claims	1-3,6-7
	No:	Claims	4,5
Industrial applicability (IA)	Yes:	Claims	1-7
	No:	Claims	

2. Citations and explanations
see separate sheet

Re Item I
Basis of the report

1. The amendments filed on 23.07.2001 do not introduce additional subject-matter, which extends beyond the content of the application as filed. Therefore, the amendments meet the requirements of Article 34(2)(b) PCT.

Re Item V
Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following documents:

   (A) Verzeletti Simona et al.: Human Gene Therapy, vol. 9, no. 15, 10 October 1998, pages 2243-2251

   (B) Database Biosis [Online] BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; August 1998 (1998-08) Mclaughlin Peter et al.:Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: Half of patients respond to a four-dose treatment program.' & JOURNAL OF CLINICAL ONCOLOGY, vol. 16, no. 8, August 1998 (1998-08), pages 2825-2833

   (C) Introna Martino et al.: 'Genetic modification of human T cells with CD20: A strategy to purify and lyse transduced cells with anti-CD20 antibodies.' Human Gene Therapy, vol. 11, no. 4, 1 March 2000 (2000-03-01), pages 611-620

2. Document C, cited in the Search Report as intermediate document, is not to be considered as state of the art, as the date of priority is validly claimed for the relevant parts of the present application.

3. Novelty

3.1 The subject-matter of claims 1-3, 6-7, relating to the use of antibodies for the treatment of the graft versus host disease and human T lymphocytes, is new in the sense of Article 33(2) PCT, because this subject-matter being related to antigens expressed on the surface of B-lymphocytes and not present on human normal T lymphocytes, is not disclosed in the prior art.

3.2 Claim 4 relates to a vector for the transfection of human T-lymphocytes with antigens expressed on the surface of B-lymphocytes and not present on human normal T-lymphocytes. this subject-matter is already disclosed in document A and therefore, claim 4 is not new in the sense of Article 33(2) PCT.

Document A discloses several vectors for the transfection of human T-lymphocytes having a cell surface selectable marker. Such improved vectors would overcome the difficulties and problems that arise after administration of a therapeutic infusion of donor lymphocytes to patients suffering from severe graft-versus-host disease (see abstract). The fact that ,the vectors of the present application are "suitable for" tranfection of human T-lymphocytes with antigens expressed on the surface of B-lymphocytes and not present on human normal T-lymphocytes, does not change the vector as such. Moreover, this vector is not characterised by means of technical features.

The additional features of claim 5 are not disclosed in document A and therefore claim 5 is novel (Article 33(2) PCT).

4. Inventive step

4.1 Document B discloses the **use of antibodies against CD20 antigen** for the preparation of compositions for the **treatment of lymphoma** (see abstract).

The differences between the subject-matter of claim 1 and the disclosure of document B, is the fact that the antibodies are used for treating **graft versus host disease** in patients who have received T lymphocytes transduced with an exogenous surface antigen and that these **antigens are not present on human normal T lymphocytes**.

Leukaemia and **lymphomas** belong to the group of haematologic neoplasias. A therapeutic role for these disorders has been assigned for **many years** to the transplantation procedures with total bone marrow or with circulating purified precursors, as **T lymphocytes**. The clinical efficacy of such procedures is based upon a mechanism of immune recognition of the leukaemia cells of the host by the donor's T Lymphocytes (Graft Versus Leukaemia). Transplants are characterised by many toxic effects including the immunologic reactivity of the donor's lymphocytes themselves against the normal tissues of the host (**Graft versus host disease**).

Document A refers that **graft versus host disease,** associated with the therapeutic infusion of donor lymphocytes, transfected with retroviral vectors carrying the HSV-tk suicide gene and a cell surface marker, after allogenic marrow transplantation, can be efficiently controlled by expression of the herpes simplex virus thymidine kinase (see abstract).

There is no suggestion in document A to use surface antigens and antibodies against them to control the activity of donor T-lymphocytes. Even combining the teachings of A and B, the skilled person would not have arrived at the claimed invention in an obvious manner. There is no reference in document B to either transduction of T-lymphocytes with exogenous CD20 or to a method for controlling GvHD using CD20-transduced T-lymphocytes. Moreover, in document B the cells involved are B-lymphocytes, not T-lymphocytes, and a person skilled in the art would have refrained from using an antibody specific for a certain antigen to treat a disease in which cells that do not express the same antigen are involved.

Thus, even combining the disclosures set out in documents A and B, the skilled person would not arrive in an obvious way to the features of claim 1. Therefore, claim 1 is based on an inventive concept as required by Article 33(3) PCT.

4.2 Claim 6 relates to human T lymphocytes transduced with antigens expressed on the surface of B-lymphocytes and not present on human normal T-lymphocytes. Considering the reasoning given in 4.1, this claim is also considered to be based on an inventive concept (Article 33(3) PCT).

For the same reasons, dependent claims 2, 3 and 7, relating to anti CD20 antibody,

are also inventive as the claims on which they depend are considered to be based on an inventive concept (Article 33(3) PCT).

4.3 The additional feature of claim 5, also relating to anti CD20 antibody, is known from document B (see abstract) and therefore this claim is not based on an inventive concept (Article 33(3) PCT). In fact, it would be obvious for the skilled person to combine the disclosures of documents A and B and arrive in this way to the features of claim 5, as both documents relate to the treatment of lymphoma or of graft versus host disease.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
SCB566PCT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 00/ 05212	07/06/2000	11/06/1999
Applicant		
CONSIGLIO NAZIONALE DELLE RICERCHE		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established by this Authority to read as follows:

   USE OF ANTIBODIES AGAINST CD20 FOR THE TREATMENT OF THE GRAFT VERSUS HOST DISEASE

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PC[redacted] 00/05212

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/395 A61K35/28 C12N5/10 C12N15/85 A61P43/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
MEDLINE, WPI Data, EPO-Internal, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VERZELETTI SIMONA ET AL: "Herpes simplex virus thymidine kinase gene transfer for controlled graft-versus-host disease and graft-versus-leukemia: Clinical follow-up and improved new vectors." HUMAN GENE THERAPY, vol. 9, no. 15, 10 October 1998 (1998-10-10), pages 2243-2251, XP000946824 ISSN: 1043-0342 abstract page 2244, left-hand column, line 18-37,51-56 page 2244, right-hand column, line 25-54 ---	4,6
A	WO 97 45142 A (GENETIC THERAPY INC) 4 December 1997 (1997-12-04) page 6, paragraph 2 -page 7, line 3 ---	1-7
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 October 2000	20/10/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Covone, M

3

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 42824 A (CELLFACTORS PLC ;DAVIES ALISON MIRIAM (GB)) 1 October 1998 (1998-10-01) page 2, line 8-15 page 8, line 14-19 claims 1-13	1-7
A	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; August 1998 (1998-08) MCLAUGHLIN PETER ET AL: "Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: Half of patients respond to a four-dose treatment program." Database accession no. PREV199800407092 XP002149247 abstract & JOURNAL OF CLINICAL ONCOLOGY, vol. 16, no. 8, August 1998 (1998-08), pages 2825-2833, ISSN: 0732-183X	1-7
P,X	INTRONA MARTINO ET AL: "Genetic modification of human T cells with CD20: A strategy to purify and lyse transduced cells with anti-CD20 antibodies." HUMAN GENE THERAPY, vol. 11, no. 4, 1 March 2000 (2000-03-01), pages 611-620, XP000946823 ISSN: 1043-0342 the whole document	1-7

3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 00/05212

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9745142 A	04-12-1997	AU 719930 B	18-05-2000
		AU 3079697 A	05-01-1998
		CA 2255941 A	04-12-1997
		EP 0977595 A	09-02-2000
		NO 985522 A	25-01-1999
WO 9842824 A	01-10-1998	AU 6736598 A	20-10-1998